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Original Article

Conjugative Transfer of Plasmid-Located Antibiotic Resistance Genes Within the Gastrointestinal Tract of Lesser Mealworm Larvae, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae)

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Abstract

The frequency of conjugative transfer of antimicrobial resistance plasmids between bacteria within the gastrointestinal tract of lesser mealworm larvae, a prevalent pest in poultry production facilities, was determined. Lesser mealworm larvae were exposed to a negative bacterial control, a donor *Salmonella enterica* serotype Newport strain, a recipient *Escherichia coli*, or both donor and recipient to examine horizontal gene transfer of plasmids. Horizontal gene transfer was validated post external disinfection, via a combination of selective culturing, testing of indole production by spot test, characterization of incompatibility plasmids by polymerase chain reaction, and profiling antibiotic susceptibility by a minimum inhibitory concentration (MIC) assay. Transconjugants were produced in all larvae exposed to both donor and recipient bacteria at frequencies comparable to control *in vitro* filter mating conjugation studies run concurrently. Transconjugants displayed resistance to seven antibiotics in our MIC panel and, when characterized for incompatibility plasmids, were positive for the N replicon and negative for the A/C replicon. The transconjugants did not display resistance to expanded-spectrum cephalosporins, which were associated with the A/C plasmid. This study demonstrates that lesser mealworm larvae, which infest poultry litter, are capable of supporting the horizontal transfer of antibiotic resistance genes and that this exchange can occur within their gastrointestinal tract and between different species of bacteria under laboratory conditions. This information is essential to science-based risk assessments of industrial antibiotic usage and its impact on animal and human health.

Introduction

The emergence of multidrug-resistant foodborne pathogens, such as *Salmonella* and *Escherichia coli*, is of major concern to human and animal health officials. Horizontal transmission of genes among bacteria, including antimicrobial resistance genes, can occur via transduction, transformation, or conjugation. The spread of antimicrobial resistance genes is affected by whether the genes reside upon mobile genetic elements within the donor bacteria, the host range of the elements, and their compatibility with other plasmids or transposons in the recipient bacteria. Conjugative transfer, which requires cell-to-cell contact, is regarded as an important method of gene flux due to the broad host range of bacteria that can participate. Understanding the epidemiology of horizontal gene transfer in an ecosystem is vital to assessing the risk for transfer of antibiotic resistance genes.

Bacterial plasmids are stable, nonessential, extra-chromosomal DNA that replicate autonomous of the bacterial cell's chromosome. Plasmids range in complexity, size, and copy number. Despite their nonessentiality, the inheritance of naturally occurring plasmids occurs with high fidelity. The intrinsic property of plasmid incompatibility behavior is used for a formal scheme of classification and identification (Novick, 1987; Couturier *et al.*, 1988). Plasmids that destabilize the inheritance of one another over time will not coexist in a cell, therefore, two plasmids that can exist in the same cell, are said to be in different incompatibility (Inc) groups, such as N and A/C (Carattoli *et al.*, 2006). Since plasmids can carry antimicrobial resistance genes and are promiscuous in terms of host

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range, they are participants in the spread of antimicrobial resistance among bacteria.

Antibiotic intervention and prophylactic antibiotic treatment are critical tools used in medical and agricultural communities, for therapeutic uses and as antimicrobial growth promotants. However, low-level continuous administration of antimicrobials may contribute to the accelerated emergence of resistance carrying zoonotic bacteria (Salyers and Amabile-Cuevas, 1997; Molbak, 2004; Anonymous, 2005). Bacteria adapt and evolve via gene transfer (Cohen, 1996), and this movement of genetic material occurs between microbes in different spatial habitats and of varied phylogenetic groups (Mazodier and Davis, 1991; Tschape, 1994). Insects are found in both medical and agricultural settings and they carry a large and varied microbial community within their gut (Cazemier *et al.*, 1997; Kaufman *et al.*, 2000; Dillon and Dillon, 2004). Transconjugation between bacterial strains inhabiting the insect gut has been previously reported (Watanabe *et al.*, 1998; Watanabe and Sato, 1998). Thus, insects must be considered potential participants in the unwanted transfer of antibiotic resistance genes.

Insects are a common occurrence in established broiler houses, and one of the most abundant insect species recovered from broiler chicken and turkey litter samples is the lesser mealworm, *Alphitobius diaperinus* (Panzer). All life stages of this insect can be found inhabiting and feeding within the litter of commercial poultry operations, which is a dynamic, nutrient-rich environment for bacterial growth (Pfeiffer and Axtell, 1980; Stafford *et al.*, 1988; Axtell and Arends, 1990; Rueda and Axtell, 1997). These insects are omnivorous scavengers and are in turn, fodder for chickens that are also in continuous contact with litter (Pfeiffer and Axtell, 1980; Axtell and Arends, 1990; Axtell, 1994; Rueda and Axtell, 1997). Thus the lesser mealworm represents a site for the potential transfer of genes amongst various microbes inhabiting the facility. Lesser mealworm beetles have been implicated in the transmission of several disease agents, including bacterial pathogens (De las Casas *et al.*, 1972, 1973, 1976; Despins *et al.*, 1994; McAllister *et al.*, 1994, 1995, 1996; Watson *et al.*, 2000).

Epidemiological and mechanistic studies of the prevalence and spread of antimicrobial resistance genes within the poultry production environment are needed. Crippen *et al.* (2008) assessed the concentration and time thresholds for the oral acquisition of *Salmonella* by lesser mealworm beetles and demonstrated that, given favorable conditions, the insects are capable of rapidly acquiring *Salmonella* from relatively low levels of environmental contamination. The purpose of the present study was to determine if *Salmonella* carrying plasmid replicons containing antibiotic resistance genes could mobilize resistance genes by conjugation between bacteria within the gastrointestinal tract of lesser mealworm larvae.

Materials and Methods

Lesser mealworms

The colony of *A. diaperinus* started at the U.S. Department of Agriculture (USDA/ARS/SPARC) has been previously described (Crippen and Sheffield, 2006). Beetles were reared in plastic containers (15×15×30 cm) with screen tops, containing 250 g of wheat bran (Morrison Milling Co., Denton, TX). Additionally, each cage contained a 6-cm^2 sponge moistened with deionized water and a 0.5-cm-thick slice of a medium-sized apple replenished twice per week; 25 g of fishmeal (Omega Protein, Inc., Hammond, LA) was added to the wheat bran once per week. The colony was held at 30°C in an 8:16 hour (light/dark) photoperiod.

Bacterial isolates

The isolates used were a multidrug-resistant AmpC *Salmonella enterica* serotype Newport isolate SN11 (donor) and an *Escherichia coli* strain JM109 (recipient) that have been previously described (Poole *et al.*, 2006a). SN11 is positive for the plasmid replicons N and A/C by polymerase chain reaction (PCR)-based replicon typing and displays antimicrobial resistance (including gene bla_{CMY-2} that confers resistance to expanded-spectrum cephalosporins) to nine antimicrobials: amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline. Tetracycline was used as the selective agent during growth. JM109 is positive for chromosomal replicons FIA, FIB, and FIC and is resistant to nalidixic acid and rifampicin, which were used as the selective agents. Overnight cultures of the bacteria were centrifuged, washed, resuspended in phosphate-buffered saline (PBS), and adjusted to approximately 10^7 colony-forming units per milliliter (CFU/mL) by spectrophotometry (absorbance at 600 nm) for subsequent conjugation experiments. Final bacterial inoculum concentration, was enumerated by serial dilution plating.

Experimental design

Ten milliliters of PBS or bacteria were added to the surface of brain heart infusion agar (BHIA; Difco, Detroit, MI) in 17×100 mm (14 mL) sterile exposure tubes (BD Biosciences, San Jose, CA), and the exposure tube was dried for 1 hour at room temperature before placing two larvae in each tube. Larvae of 3–4 weeks of age were selected from the colony, placed in the tube, and allowed to move freely and presumably feed in the tubes, which were held at 30°C in the dark. Three replications of each experimental exposure protocol were conducted using at least 10 larvae per treatment group.

The insects were surface disinfected after exposure to treatment bacteria to assure no bacteria remained on the exterior of the larvae. Two different surface disinfection protocols were used. Protocol-1 surface disinfection consisted of a 1-minute immersion in SporGon® (Decon Labs, Inc., Bryn Mawr, PA), followed by transfer to an empty, sterile containment well at room temperature for 2 minutes while still coated with SporGon. The efficacy of the disinfection was validated by subsequent exposure of the washed larvae to brain heart infusion broth (BHIB; Difco) for 1–2 minutes. The larvae were then dried by blotting on sterile absorbent paper and utilized. The BHIB was incubated overnight at 37°C, followed by plating on BHIA, incubation overnight at 37°C, and enumeration. Any larvae found to be externally contaminated with bacteria after surface disinfection were removed from the study. Protocol-2 surface disinfection consisted of a sequential immersion in 95% ethanol and SporGon, as previously described (Crippen and Sheffield, 2006). The efficacy of the disinfection was validated as described above.

The four treatment groups were as follows: 1) a control (CNTL) group exposed only to PBS-treated tubes for 16 hours; 2) a donor only (DO) group exposed to a mean of

$1.4\times10^8 \pm 9.4\times10^7$ CFU/mL SN11 for 2 hours, surface disinfected (protocol-1), and then transferred to PBS-treated tubes for 16 hours; 3) a recipient only (RO) group exposed to a mean of $9.3\times10^6 \pm 7.7\times10^6$ CFU/mL JM109 for 16 hours then surface disinfected (protocol-2); and 4) a presumptive transconjugant group (TR) exposed to SN11 for 2 hours, surface disinfected (protocol-1), and then exposed to JM109 for 16 hours and surface disinfected (protocol-2). The experimental design for the treatment groups is shown in Table 1. After treatment, larvae were harvested from the exposure tubes and placed individually into 1.2 mL of BHIB, homogenized, and enriched by incubation at 37°C for 16 hours. When frequency determinations were made, homogenized larvae were immediately plated by serial dilution without enrichment.

Isolation on selective media

An aliquot (100 μL) from each homogenate in the CNTL, DO, and RO treatments was plated in duplicate onto each of three plate types: MacConkey agar (Becton Dickinson, Sparks, MD) containing 32 μg/mL each of nalidixic acid and rifampicin (MAC-NR); MacConkey agar containing 32 μg/mL tetracycline (MAC-T); and MacConkey agar containing 32 μg/mL nalidixic acid, rifampicin, and tetracycline (MAC-NRT). An aliquot (100 μL) from each homogenate in the TR was plated onto four to six MAC-NRT plates. Initially a single colony based on phenotypic characteristics was selected from each of the plates for subsequent characterization.

Isolated colonies were selected from MAC-T and MAC-NR plates from groups DO and RO for subsequent validation as experimental bacteria (SN11 and JM109) and from each of the MAC-NRT plates in group TR for subsequent validation as putative transconjugants. Each colony was subcultured onto BHIA and incubated at 37°C for 16 hours and subcultured a second time by transfer onto a trypticase soy agar plate with 5% sheep blood (TS-blood agar; BVA Scientific, San Antonio, TX) and a BHIA plate. The bacteria from these isolation plates were used for indole production testing, antibiotic susceptibility testing, and molecular characterization of replicons. If this analysis resulted in a negative finding for experimental or putative transconjugant bacteria, another colony from the same plate was selected for analysis.

Filter mating control bacterial conjugation

For each experiment, the same overnight SN11 and JM109 preparations were used for *in vitro* conjugation mating on solid support filters in triplicate as previously described (Bischoff *et al.*, 2004; Poole *et al.*, 2006b). TR bacterial cultures were mixed in a 1:10 ratio (donor/recipient) by combining 0.05 mL of donor culture and 0.50 mL of recipient culture in 4.5 mL of BHIB. Each mixture was passed through a 0.45 μm, 13-mm filter (Millipore, Billerica, MA) and placed on a TS-blood agar plate then incubated 16–18 hours at 30°C. DO and RO bacterial cultures were processed identically using a 1:10 ratio (donor/PBS) and 1:10 ratio (PBS/recipient), respectively. The filters were submerged and agitated in 1.0 mL BHIB and 100 μL aliquots of the DO, RO, and TR groups were plated in duplicate on MAC-T and MAC-NR plates and in quadruplicate onto MAC-NRT plates then incubated 16–18 hours at 37°C and analyzed.

Table 1. Schematic of Larvae Exposure Design for PBS (CNTL), Donor Only (DO), Recipient Only (RO), or the Presumptive Transconjugant (TR) Group

	Bacterial exposure (hours)				
	SN11[a]		*PBS*	*JM109*[b]	
CNTL	—	—	16	—	—
DO	2	SD-1[c]	16	—	—
RO	—	—	—	16	SD-2[d]
TR	2	SD-1	—	16	SD-2

[a]*Salmonella enterica* serotype Newport isolate.
[b]*Escherichia coli.*
[c]Surface disinfection by protocol 1.
[d]Surface disinfection by protocol 2.

Determination of transconjugant frequency

The number of experimental bacteria in the larvae or on the filter was determined by serial dilution plating of the DO or TR group homogenates onto MAC-NR and MAC-NRT plates, respectively. The plates were incubated overnight at 37°C and enumerated. The transfer frequency of plasmids from donor to recipient cells was calculated as the number of transconjugant colonies on MAC-NRT divided by the number of recipient colonies on MAC-NR. Three replicate experiments were performed to establish a mean.

Determination of indole production

Each colony selected from the BHIA isolation plate was tested for indole production using 1% p-dimethylaminocinnamaldehyde (Indole Reagent; Anaerobe Systems, Morgan Hill, CA) per the manufacturer's instructions.

Determination of antibiotic susceptibility

The antimicrobial minimum inhibitory concentration (MIC) for growth was determined by broth microdilution standards of the Clinical Laboratory Standards Institute (CLSI, 2003). Antibiotic susceptibility of bacteria from the TS-blood agar isolation plate was performed using a Sensititre® automated antimicrobial susceptibility system according to the manufacturer's instructions (Trek Diagnostic Systems, Westlake, OH) and a National Antimicrobial Resistance Monitoring System (NARMS) panel for Gram-negative enteric bacteria (CMV1AGNF, Trek Diagnostic Systems, Cleveland, OH). The following antimicrobials were evaluated on the CMV1AGNF 96-well plate (breakpoint for resistance): amikacin (≥64), amoxicillin/clavulanic acid (≥32/16), ampicillin (≥32), cefoxitin (≥32), ceftiofur (≥8), ceftriaxone (≥64), chloramphenicol (≥32), ciprofloxacin (≥4), gentamicin (≥16), kanamycin (≥64), nalidixic acid (≥32), streptomycin(≥64), sulfisoxazole (≥256), tetracycline (≥16), and trimethoprim/sulfamethoxazole (≥4/76). Rifampicin (≥256) (Sigma Aldrich Corp., St. Louis, MO) susceptibility was determined manually by broth microdilution using the methods described by the CLSI (2003). The following ATCC strains were used as controls for antimicrobial susceptibility testing: *E. coli* 25922, *Enterococcus faecalis* 29212, *Staphylococcus aureus* 29213, and *Pseudomonas aeruginosa* 27853 (American Tissue Culture Collection, Manassas, VA).

Data were interpreted using CLSI breakpoints (CLSI, 2005).

Replicon characterization by PCR-based replicon typing

PCR-replicon typing of SN11, JM109, and transconjugant *E. coli* isolates was performed using the primers and methods of Carattoli *et al.* (2005) divided into three multiplex panels as described by Johnson *et al.* (2007). The 18 replicons examined included B/O, K/B, FIIA, FIA, FIB, FIC, HI1, HI2, Y, I1, Frep, X, L/M, N, P, W, T, and A/C. Positive controls used in the procedure were generously provided by A. Carattoli (Instituto Superiore di Sanita, Rome, Italy). The replicon control DNA was transformed into *E. coli* DH5α subcloning efficiency competent cells (Invitrogen, Carlsbad, CA).

For rapid screening, template DNA was prepared by a boiling lysis procedure. Briefly, a 10 μL loop full of bacteria from the BHI isolation plate for each larva was resuspended in 0.5 mL of ultrapure H_2O and brought to 100°C for 15 minutes then centrifuged for 1 minutes and frozen at −20°C until analysis. Multiplex PCR amplifications (50 μL) contained template DNA (100 ng), 25 pmol of each of the selected primers, 25 μL of HotStarTaq Mastermix (Qiagen, Valencia, CA), and ultrapure water *q.s.* For amplification, the reaction mixture was first heated to 95°C for 10 minutes, followed by 35 cycles of 1 minute at the denaturation temperature of 94°C, 1 minute at the annealing temperature of 58°C, 2 minutes at the extension temperature of 72°C, and concluded with a single final extension of 5 minutes at 72°C. Amplicons were visualized on 1% Tris-acetate-EDTA agarose gels alongside size markers, 100 bp and 1 kb ladders (NewEngland Biolabs, Ipswich, MA), and appropriate purified control replicons. If an amplicon of expected size was observed, the isolate was considered positive for that replicon.

Statistical analysis

Three replicate experiments evaluating 10 larvae per RO and 11, 12, and 18 larvae per TR group, respectively, were established for analysis of conjugation frequencies. Descriptive statistics were generated and presented in table formats (Microsoft Office Excel 2003, Microsoft Corp., Redmond, WA). Data were analyzed using commercially available statistical software (JMP version 5.0.1, SAS Institute Inc., Cary, NC). A comparison of differences in median conjugation frequency between experiments was performed by nonparametric Kruskall–Wallis test and chi-square comparison (Agresti, 2002).

Results

Surface disinfection protocols used in our study removed external bacteria from the insect larvae and ensured that resulting conjugation was measured from an internal source only. Protocol-1 is used because larvae survive the procedure and can be exposed to a subsequent treatment. The disinfection efficacy for protocol-1 was 97%. Protocol-2 surface disinfection consisted of a sequential immersion in 95% ethanol then SporGon, as previously described (Crippen and Sheffield, 2006). The larvae do not survive exposure to 95% ethanol, but the efficacy of disinfection by protocol-2 is 100% in beetles as previously reported (Crippen and Sheffield, 2006).

Determination of production of transconjugants

Because these studies were not done with gnotobiotic beetles, some bacteria native to the insects' gut was expected to grow on the selective media plates. Therefore, initial colony selection for experimental bacteria from the homogenates was made by selective plating and phenotypic characterization. All larval homogenates in the DO group produced donor bacterial growth on MAC-T plates and recipient bacterial growth on MAC-NR plates (Table 2). All larval homogenates from the TR group produced presumptive transconjugants on MAC-NRT. No growth of donor occurred on MAC-NR or MAC-NRT, and no growth of recipient occurred on MAC-T or MAC-NRT. There was a low incidence of tetracycline-resistant, Gram-negative bacterial growth from the CNTL and RO groups on MAC-T plates. However, further biochemical and molecular analysis determined that these were not experimental donor or recipient bacteria and were considered to be flora originating from the larval gut.

Presumptive colonies, selected based on phenotypic characteristics, were subsequently characterized by biochemical and molecular analyses. The indole spot test is a biochemical analysis to determine the ability of the bacteria to cleave indole from the amino acid tryptophan. Donor SN11 was unable to produce indole, but recipient JM109 and putative transconjugant *E. coli* did produce indole. Sixty indole-negative colonies were analyzed from the DO group. Sixty and 128 indole-positive colonies were analyzed from RO and TR groups, respectively. Colonies from the corresponding groups from the filter mating were also indole characterized.

The selected colonies were next evaluated for antibiotic susceptibility. Extensive preliminary studies consistently demonstrated a high frequency of donor or recipient bacterial growth on the corresponding selective media plates (data not shown). Therefore after characterization for indole production, only three colonies from MAC-T plates for group DO and three from MAC-NR plates for group RO for each experiment were evaluated further. Four putative transconjugant colonies from each larva of the TR group were further characterized ($n = 112$).

Table 2. Results of Plate Scoring from Selective Plating of Filter Conjugation and of Lesser Mealworm Larvae Fed PBS (CNTL), Donor Only (DO), Recipient Only (RO), or Both Donor and Recipient (TR) Bacteria

			% of plates with growth		
	Treatment group	*No. of samples*	*MAC-T*	*MAC-NR*	*MAC-NRT*
Larvae	CNTL	30	27[a]	0	0
	DO	30	100	0	0
	RO	30	25[a]	100	0
	TR	28[b]	ND	ND	100
Filter	CNTL	1	0	0	0
	DO	1	100	0	0
	RO	1	0	100	0
	TR	1	ND	ND	100

[a]Further analysis determined that bacteria recovered was not SN11 or JM109.

[b]Two larvae found to be positive after external disinfection were removed from further analysis.

MAC, MacConkey agar; T, tetracycline, N, nalidixic acid; R, rifampicin; ND, not done.

Table 3. Results of Replicon Profiling and Antibiotic Susceptibility Characterization of Filter Conjugation and of Lesser Mealworm Larvae Fed PBS (CNTL), Donor Only (DO), Recipient Only (RO), or Both Donor and Recipient (TR) Bacteria[a]

	DO	*RO*	*TR*
Replicon profile[b]			
A/C	+	−	−
FIA	−	+	+
FIB	−	+	+
FIC	−	+	+
N	+	−	+
Antibiotic susceptibility			
Amoxicillin/clavulanic acid	+	−	−
Ampicillin	+	−	+
Cefoxitin	+	−	−
Cetiofur	+	−	−
Ceftriaxone	+	−	−
Chloramphenicol	+	−	+
Nalidixic acid	−	+	+
Rifampicin	−	+	+
Streptomycin	+	−	+
Sulfisoxazole	+	−	+
Tetracycline	+	−	+

[a]+, plasmid present or resistant to antimicrobial; −, plasmid absent or susceptible to antimicrobial.
[b]A/C, FIA, FIB, FIC, and N represent different plasmid incompatibility groups.

The recipient JM109 isolate contains two antimicrobial resistance tags and the donor SN11 isolate contains nine resistance tags. The resulting putative transconjugant bacteria contained seven resistance tags consisting of ampicillin, chloramphenicol, nalidixic acid, rifampicin, streptomycin, sulfisoxazole, and tetracycline (Table 3). Not all of the donor resistance phenotypes transferred during conjugation. Every larva ($n = 28$) produced a colony of expected experimental transconjugants. Ninety-three percent of the colonies characterized displayed the TR susceptibility profile of seven resistance tags. The same phenotype was displayed by all ($n = 4$) of the filter mating TR group.

Molecular analysis by PCR-based replicon typing confirmed that DO groups contained the replicons A/C and N; and RO groups contained the replicons FIA, FIB, and FIC. The resulting TR bacteria contained the replicons FIA, FIB, FIC, and N (Table 3). The A/C replicon was not transferred from donor SN11 to recipient JM109. Every larva ($n = 28$) produced a colony of expected experimental transconjugants. Of the 112 indole-positive colonies taken from selective media, 94% of the colonies characterized displayed the expected transconjugant replicon profile, 93% displayed the expected transconjugant antibiotic susceptibility, and 88% displayed both the same susceptibility and replicon profile as the transconjugants produced by filter mating.

Determination of transconjugant frequency

Frequency measurements demonstrated an average internal acquisition of 2.8×10^5 CFU/larva of JM109 over the 18-hour exposure period (Table 4). We did not differentiate between ingested bacteria and those that might be present due to proliferation for determining "Recipient only mean Acquisition" because all would be available for possible conjugation. The mean conjugation frequency found *in vivo* in larvae was not significantly different ($p < 0.01$) from that produced by the *in vitro* filter mating, but showed greater fluctuation in transconjugant production ranging from 1×10^{-1} to 2×10^{-3} CFU/larva, versus 6×10^{-2} to 9×10^{-3} CFU/filter. No significant difference ($p < 0.01$) in conjugation frequency on filters or within larvae was found between experimental replications.

Discussion

With the emergence of multidrug-resistant pathogens, poultry producers, who have long been under pressure to limit dispersal of pathogenic bacteria on retail products, must now limit the use of antimicrobial agents (Zhao *et al.*, 2001, 2006). Comprehensive knowledge of the pathways involved in the transfer of antibiotic resistance genes within the environment remains unclear. Previous studies have investigated the role of insects in this process. However, the scope to which insects contribute to antibiotic resistance gene dispersal is difficult to establish. In order to institute viable biosecurity control measures in the brooder house environment, a more in-depth understanding of the relationship between pathogens, antibiotic resistance genes, insect vectors, and individual

Table 4. Conjugation Frequencies Measured in the Gut of the Lesser Mealworm Larvae for Donor (*S.* Newport SN11) and Recipient (*E. coli* JM109)

	Dose[a]			*Mean acquisition (CFU/larva)*[b]	
	Donor (CFU/mL)	*Recipient (CFU/mL)*	*positive/*n[c]	*Recipient only*	*Conjugation frequency*[d]
Larvae	3.90×10^7	2.20×10^7	10/10	$4.16\times10^5 \pm 2.44\times10^5$	$7.73\times10^{-2} \pm 1.97\times10^{-1}$
	1.35×10^7	1.60×10^7	14/14	$8.00\times10^4 \pm 1.01\times10^5$	$4.82\times10^{-2} \pm 1.01\times10^{-1}$
	5.77×10^7	1.58×10^6	18/18	$3.50\times10^5 \pm 3.36\times10^5$	$1.06\times10^{-1} \pm 5.46\times10^{-3}$
Filter	3.90×10^7	2.20×10^7	3/3	$1.44\times10^8 \pm 1.02\times10^8$	$6.48\times10^{-2} \pm 1.49\times10^{-2}$
	1.35×10^7	1.60×10^7	3/3	$2.07\times10^8 \pm 1.82\times10^8$	$9.49\times10^{-3} \pm 3.22\times10^{-3}$
	5.77×10^7	1.58×10^6	3/3	$1.49\times10^8 \pm 2.90\times10^7$	$6.34\times10^{-2} \pm 1.70\times10^{-2}$

[a]Agar dosed with 10 μL bacteria at this concentration level.
[b]Mean number of bacteria acquired internally by larva or measured on filters; CFU/larva or CFU/filter $\pm$ standard deviation.
[c]Number of larvae or filters positive for transconjugants/total number of larvae or filters exposed.
[d]Conjugation frequency equals the number of transconjugants produced by larva or filter divided by the number of recipient bacteria acquired by larvae or filter mating.

birds is required. The movement of foodborne pathogens facilitated by insects via mechanical dislodgement from the exoskeleton, fecal deposition, or even regurgitation, in the case of flies, has been documented; however, investigation into the occurrence of DNA exchange by horizontal transfer has been largely overlooked. One method of gene transfer of plasmid-located genes is by conjugation from donor to recipient bacteria. This transfer method allows the movement of genetic material between phylogenetically different bacterial groups within microbial communities occupying the same spatial habitat.

The capacity of the ecosystem to support horizontal gene transfer is affected by the density of bacteria present. In high bacterial density environments there is a greater opportunity for gene transfer. Arthropods, such as crickets, grasshoppers, cockroaches, and beetles, harbor large bacterial concentrations (10^8 to 10^9/mL gut) in the foregut, (10^8 to 10^{10}/mL gut) in the midgut, and (10^8 to 10^{11}/mL gut) in the hindgut (Cazemier *et al.*, 1997). We were interested in determining if plasmid transfer could occur between different bacterial groups within the confines of the gastrointestinal tract of the lesser mealworm, an insect that commonly occupies poultry house facilities. The environmental parameters which facilitate horizontal gene transfer amongst bacteria or the establishment of reservoir populations of antibiotic-resistant bacteria within poultry facilities is poorly understood. Because of the nature of their habitat and food sources, beetles and larvae can easily contaminate their external surfaces, as well as ingest bacteria. Thus, they aid in the dispersal of bacteria into the environment by direct contact, excretion of internally harbored bacteria, and as a food source to poultry.

Horizontal gene transfer occurs within facilities which support nutrient-rich habitats with high densities of bacteria. Ashelford *et al.* (2001) measured frequencies of gene transfer up to 8.4×10^{-4} within the biofilm of sewage filter beds at treatment plants and felt that seasonal temperature fluctuations affected the frequencies. Earthworms mediate transport of bacteria and enhance plasmid transfer to microorganisms within soil (Daane *et al.*, 1996, 1997), but conjugation occurring within the insect must also be considered. Armstrong *et al.* (1989) investigated the survival of specific plasmid (pBR322)-bearing bacteria when ingested by the cutworm, *Peridroma saucia*. The cutworm ingested, carried, and dispersed the bacteria by expulsion in their frass. A differential retention of bacteria in the gut based on bacterial species was found. In a series of investigations, soil microarthropods were determined to be vectors for microorganisms and their gut densely colonized with bacteria providing ideal conditions for bacterial conjugation (Hoffmann *et al.*, 1998, 1999; Thimm *et al.*, 1998). These researchers regarded the gut of the insect to be a "hot spot for gene transfer" (Hoffmann *et al.*, 1998).

Investigations in this lab have demonstrated that lesser mealworm beetles are capable of rapidly ingesting bacteria and harboring large concentrations internally (Crippen *et al.*, 2008), and this study demonstrated that the larvae are also capable of ingesting large quantities (2.8×10^5 CFU/larva) of bacteria. Indeed our results support the opinion that the alimentary tract of insects provides a habitat for bacteria in densities that may facilitate bacterial conjugation and elevate the efficiency of gene transfer. Given the relatively limited size of the alimentary tract in these larvae, it is likely that the donor and recipient bacteria are found in close proximity to one another. Because of the quantity of bacteria the larvae are capable of ingesting and the high percentage of insects (100%) which produced transconjugants, we agree their gut represents a "hot spot" for gene transfer. It is unknown whether the resulting counts were from multiple transfers or a single transfer followed by expansion of the transconjugant population through reproduction. However, the counts were compared to frequencies obtained from the ideal conditions of *in vitro* filter mating and similar values were obtained.

In one of our experiments, the conjugation frequency was found to be higher in the larvae than the filter mating controls. This could be due to growth of bacteria within the gut of the larvae. Pseudomonad bacteria were found to increase in numbers within the gut of the terrestrial isopod, *Porcellio scaber*, and survive within the feces (Clegg *et al.*, 1994). However, it could also be due to assay limitations since the *in vitro* filters are placed on top of agar and subsequent retrieval of the filter may not recover all bacteria if growth results in spread of bacteria onto the nearby agar surface.

A few studies have described gene transfer within the invertebrate alimentary tract (Armstrong *et al.*, 1987; Jarrett and Stephenson, 1990; Hoffmann *et al.*, 1998; Thomas *et al.*, 2000, 2001; Ashelford *et al.*, 2001). However, none of these investigations described methodology to disinfect the external surface of the insect prior to exposure between the donor and recipient bacteria. It is possible that plasmid transfer occurred externally and that the transconjugant was subsequently ingested. Methodology used in our study removes bacteria from the outside of the insect to assure that resulting transconjugation was measured from internal sources only. Adamo and Gealt (1996) externally disinfected *Rhabditis* nematodes by chlorine bleach treatment between bacterial exposures and found conjugation occurred within the alimentary canal. Petridis *et al.* (2006) force fed the house fly (*Musca domestica*) donor and recipient *E. coli* strains, thus eliminating the need to disinfect external surfaces of the insects and allowing calculation of exact treatment dose. They reported a plasmid transfer rate of 10^{-2} to 10^{-3} per donor cell in the gut 1 hour after feeding. However, quantitation of transconjugant concentration was done only by serial dilution on nonselective Luria–Bertani (LB) media with appropriate antibiotics. No further validation of transconjugant colonies was performed. Therefore, contamination by normal flora or horizontal transfer to other native recipients could have been included in the final counts. We too did not fully characterize every colony while performing conjugation frequency counts. However, in our experiments where multiple colony picks were fully characterized, 88% of the bacterial colonies displayed the expected experimental transconjugant profile.

Armstrong *et al.*(1990) investigated transconjugation between *Enterobacter cloacae* donor and recipient strains in the digestive tract of the cutworm (*P. saucia*) by feeding the insects in sequence on plants sprayed with recipient or donor bacteria. They concluded that transfer of recombinant DNA occurred most likely in the gut or frass, but that the events were rare. In contrast, the swapping of replicons occurred at a high frequency in our study, ranging from 2.2×10^{-3} to 1.1×10^{-1}. Watanabe and Sato (1998) and Watanabe *et al.* (1998) also

found a high frequency plasmid transfer (10^{-1} to 10^{-3} per recipient) within the digestive tract of silkworm larvae (*Bombyx mori*) between *E. cloacae* and the plant-epiphytic bacteria, *Erwinia herbicola*. *E. coli* was demonstrated by Hinnebusch *et al.* (2002) to transfer resistance to *Yersinia pestis* in the flea (*Xenopsylla cheopis*) gut. A transfer frequency of 10^{-3} was measured 3 days post infection. Four weeks post infection, 95% of the fleas contained an average of 10^3 transconjugant *Yersinia*. Ashelford *et al.* (2001) measured gene transfer within the invertebrate gnat (*Sylvicola fenestralis*), inhabiting sewage filter beds. Transconjugants were detected in 60% of the invertebrates sampled and gene transfer frequencies ranged from 3.6×10^{-6} to 1.6×10^{-2}.

In our study the A/C replicon in SN11 was nonconjugative. This was consistent with previous conjugations done with this donor and recipient pair (T.L. Poole, unpublished data). The transconjugants produced lacked resistance to cephalosporins. This was not surprising since the bla_{CMY} gene conferring resistance to the extended-spectrum cephalosporins is carried on the A/C replicon. It is likely that genes conferring resistance to ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline, which were transferred to JM109, were present on the N replicon.

In conclusion, the purpose of this study was to determine if the alimentary tract of the lesser mealworm provides an accommodating environment permitting horizontal gene transfer through plasmid conjugation of antibiotic resistance genes. Using bacteria of different genera, not only did 100% of the larvae exposed to both the donor and recipient strains produce transconjugants in their gut, but a high percentage (88%) of the colony picks were transconjugants and the conjugation frequency was similar to that of control filter mating. Understanding the dynamics of movement of pathogenic bacteria within the environment and between species is crucial to unraveling the epidemiology of bacterial diseases and to developing biosecurity measures to reduce or eliminate the unintentional propagation and dissemination of antibiotic resistance between flocks or to nearby fauna. Conditions in the poultry house environment would not restrict the lesser mealworm's movements. These insects are known to congregate in great numbers under and around feed and water stations, as well as along and in the walls of the structure (Axtell and Arends, 1990). They are a difficult pest to control and are often inadvertently dispersed to neighboring residences by the spreading of beetle-containing manure to nearby fields (Armitage, 1986; Calibeo-Hayes *et al.*, 2005). These factors make them potential participants in the dissemination of bacteria within the poultry house and into the surrounding environment. Therefore, the quantity of these insects present in a poultry house should to be considered when assessing the risk of the spread of antibiotic resistance genes. Future studies in this lab will explore the capacity of the lesser mealworm to transfer genes to the gut microflora of individual birds.

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Disclosure Statement

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